Supporting Information

RNA Imaging in Living Mouse Enabled by in Vivo Hybridization Chain Reaction Circuit with Tripartite DNA Probe

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EXPERIMENTAL SECTION

Materials. N-hydroxysulfosuccinimide sodium salt (Sulfo-NHS), N-hydroxysuccinimide (NHS), folate, dicyclohexylcarbodiimide (EDC), sodium azide (NaN_3), bathophenanthroline disulfonic acid disodium salt, copper (II) sulfate, sodium L-ascorbate, amiloride, CPZ, Me-β-CD, nystatin and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The azido-modified peptide azido-GGKKKKKKKK (N-to-C direction) was synthesized from China Peptides Co. Ltd. (Shanghai, China, 98.3% purity). DNA ladder, folate-free Gibco RPMI 1640 medium and 10000 Da molecular weight cutoff dialysis membrane were obtained from Sangon Biotech Co. Ltd. (Shanghai, China). HeLa cells (human cervical carcinoma cell line), MCF-7 cells (human breast adenocarcinoma cell line) and L-02 cells (mouse endothelial cell line) were obtained from the cell bank of Central Laboratory at Xiangya Hospital (Changsha, China). RPMI 1640 medium, DMEM high glucose medium, penicillin, streptomycin and 10% heat-inactivated fetal bovine serum (FBS), 100% fresh fetal bovine serum (FBS), lipofectmine 3000 transfection reagent, miRNA-21 mimic (sense strand: 5'-UAG CUU AUC AGA CUG AUG UUG A-3'; antisense strand: 5'-AAC AUC AGU CUG AUA AGG UAT T-3') and inhibitor (5'-UCA ACA UCA GUC UGA UAA GCU A-3') were purchased from Thermo Fisher (MA, USA). Folate receptor alpha plasmid was obtained from Genscript Co. Ltd. (Nanjing, China). LysoTracker Green DND-26 was obtained from Invitrogen (Carlsbad, CA). Oligonucleotides used in this work (Table S1), all HPLC-purified and lyophilized, were obtained by Sangon Biotech Co. Ltd. (Shanghai, China). All other chemicals were of analytical grade and purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Ultrapure water was obtained through a Millipore Milli-Q water purification system (Billerica, MA, USA) and had an electric resistance >18.25 MΩ.

Synthesis of multivalent folate probe (FAP). Folate-NHS was synthesized performed as follows[S1]: Briefly, NHS (0.26 g) and EDC (0.47 g) were added successively in a mixture of 9.75 mL DMSO and 0.25 mL triethylamine containing 0.5 g folate. The reaction mixture was stirred overnight at room temperature followed by filtration to remove the byproduct. The solution was concentrated under reduced pressure followed by addition of ethyl ether (20 mL). The resulting yellow precipitate, folate-NHS, was washed three times with ethyl ether, dried under vacuum, and stored at -20 °C for future use.

The conjugation of folate-NHS with peptide was performed as follows: In 500 μL PB buffer (0.1 M Na_2HPO_4, 0.1 M KH_2PO_4, pH 7.4) containing 10 mM folate-NHS, 5 μL aqueous solution of the poly-lysine peptide azido-GGKKKKKKKK (10 mM) was added. The mixture was stirred at 37 °C for 2 h, and the resulting folate-conjugated peptide solution (100 μM) was purified by HPLC on a reverse phase C-18 column (50% acetonitrile, 0.1% trifluoracetic acid) to remove the excessive folate. The

folate-conjugated peptide was confirmed using ESI MS (Fig. S1), and the MS spectrum was obtained in a positive ion mode on an LTQ OrbitrapVelos Pro mass spectrometer (Thermo Fisher Scientific).

The conjugation of the folate-conjugated peptide and the 5'-alkynyl modified ssDNA Linker L was performed by using the click chemistry reaction in HBS buffer (10 mM HEPES, 145 mM NaCl, pH 6.5). In a mixture containing 17 μL copper (II) sulfate pentahydrate solution (1.5 mM), 19 μL sodium L-ascorbate solution (30 mM), 11 μL bathophenanthroline disulfonic acid disodium salt hydrate solution (5 mM) and 57.5 μL folate-conjugated peptide (100 μM), 115 μL Linker L solution (20 μM) was added to the final concentration 4 μM. The reaction mixture was stirred for 1 h with the protection of N_2. The resulting folate-peptide conjugated FAP probe was purified by dialyzed using 10000 Da molecular weight cutoff dialysis membranes against sterile water in order to remove the excessive folate-peptide. Fresh sterile water was changed every 2 h in 2 days. The product was confirmed by HPLC and UV-vis absorption spectroscopy (Fig. S2-S3). The UV-vis absorption spectra were measured in a quartz cell with an optical length of 1 cm using a UV-2550 spectrometer (Shimadzu, Japan) with a wavelength interval of 2 nm. HPLC was performed on a reverse phase C-18 column (50% acetonitrile, 0.1% trifluoracetic acid) with UV-vis absorption detection (Agilent Technologies, Germany).

Preparation of Y-shaped scaffold, tripartite DNA probe and gel electrophoresis analysis. The Y-shaped DNA was prepared by hybridization of three ssDNA, Ya, Yb and Yc (Table S1), with an equal concentration in TAE-Mg^{2+} buffer (40 mM Tris buffer, 2 mM EDTA, and 12.5 mM $MgAc_2$) to the final concentration 4 μM.

The one-pot synthesis of the tripartite DNA nanostructure probe was performed by mixing three ssDNA, Ya, Yb and Yc with an equal concentration (2 μM) in TAE-Mg^{2+} buffer followed by the addition of an equal volume of the mixture of hairpin probes H1, H2, and FAP probe with the same

concentration of 2 μM in TAE-Mg^{2+} buffer. The resulting tripartite DNA nanostructure probe (1 μM) was kept at -20 °C for future use.

In gel electrophoresis analysis, ~1 μM DNA probe or a mixture of DNA probe with target miR-21 was loaded into a well of the precast 4% agarose gel. The reaction time was 3 hours. Electrophoresis was run at 100 V in 1×TBE buffer for 60 min. The DNA ladder was used as the molecular weight standard. After electrophoresis, the gel was visualized using a Tocan 240 gel imaging system (Tocan Biotechnol. Co., Shanghai, China).

The estimated yield of Y-DNA was calculated by Fiji software (https://fiji.sc/). The yield of Y-DNA was the integral intensity ratio of the main band to all the bands.

In vitro detection of RNA target. The sample containing miR-21 target of a given concentration was mixed with 100 nM Y-H1-H2-FAP probe or Y-H1-FAP probe in the TAE-Mg^{2+} buffer. The mixture was incubated at 37 °C for 3 hours to complete the reaction.

The fluorescence spectra were recorded at room temperature in a quartz cuvette on a FluoroMax-4 spectrofluorometer (HORIBA, NJ, USA). The excitation wavelength was 635 nm and the emission wavelengths were in the range from 650 nm to 750 nm with both excitation and emission slits of 5 nm under a PMT voltage of 950 V. Time-dependent fluorescence monitoring was performed with a time interval 20 s in a 96-well microplate on a Tecan Infinite M-1000 microplate reader. The excitation wavelength was 635 nm, and the emission wavelength was 664 nm with both excitation and emission bandwidths of 5 nm.

Cell culture and confocal imaging. HeLa cells and L-02 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 g/mL streptomycin. MCF-7 cells were grown in Dulbecco's-modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 g/mL streptomycin. All cell lines were maintained at 37

°C in a humidified incubator containing 5% CO_2. The cell density was determined using a TC20 automated cell counter (BIO-RAD, USA).

To obtain MCF-7 and L-02 cells with folate receptor expression on the membrane and modulate the expression of miR-21 in the cells, transfection experiments were performed as follows: Folate receptor alpha plasmid (1 μg) was transfected into the cells by using lipofectamine 3000 transfection reagent. Synthetic miRNA-21 mimic or inhibitor of a given concentration was also transfected with lipofectamine 3000. After transfection over 24 h, the cells were subjected to further analysis.

Cells were plated on a 35 nm Petri dish with 14 mm well and grown to 50–70% confluency in 2 mL corresponding medium (no folate) at 37 °C for 24 h. After incubated with 1 mL culture medium (no folate) containing 100 nM Y-H1-H2-FAP probe or control probe at 37 °C for 3 h followed by washing three times with cold PBS, the cells were incubated with 1 mL fresh medium at 37 °C.

Fluorescence imaging of intracellular localization was performed as follows: cells plated on a 35 mm Petri dish were incubated with 1 mL culture medium (no folate) containing 100 nM Y-H3-H4-FAP probe or Y-H1-H2-FAP probe at 37 °C. After washing three times with cold PBS, the cells were incubated with fresh RPMI 1640 medium containing 100 nM Lysosome traker (Lyso@traker green) for 20 min followed by imaging.

For cellular uptake experiment, cells grown on a 35 mm Petri dish were firstly incubated for 1 h at 37 °C with 1 mL culture medium containing 1% NaN_3, nystatin (50 μM), chlorpromazine (50 μM) amiloride (50 μM) or methyl-β-cyclodextrin (10 mM). Then, the cells were incubated with 1 mL culture medium containing 100 nM Y-H3-H4-FAP probe and the inhibitor of the aforementioned concentration at 37 °C for 1 h followed by imaging.

All fluorescence images were obtained using an oil immersion objective (100×, NA 1.3) on a Nikon TI-E+A1 SI confocal laser scanning microscope (Japan). Red laser (640 nm) was used for Cy5

fluorescence detection, a laser (488 nm) was used as the excitation source for the detection of LysoTraker Green DND-26, a laser (560 nm) was used as the excitation source for the detection of the Y-H3-H4-FAP probe.

Flow cytometry assay of miRNA-21 expression using DNA probe. HeLa, MCF-7 and L-02 cells (10^5 cells) were incubated with 100 nM of the specified probe in 1 mL fresh medium at 37 °C for 3 h followed by washing three times with cold PBS. The cells were treated with 50 μL of 0.25% trypsin for 5 min and centrifuged for 2 min at 2000 g followed by two washes with 500 μL PBS and resuspension in 1 mL PBS for flow cytometry assay on a CytoFLEX™ flow cytometer (Beckman coulter, USA).

Quantitative Reverse transcription-PCR (qRT-PCR) analysis of miRNA in cells. Total cellular RNA was extracted from different cells such as HeLa, MCF-7 and L-02 cells using the RNeasy Mini Kit (Qiagen, USA) according to the indicated protocol. The cDNA samples were prepared by using reverse transcription (RT) reaction with an iScript kit (Bio-Rad, USA) according to its manual. The cDNA samples were store at -20 °C for future use. qPCR analysis of cDNA was performed with SybrGreen PCR Master Mix (ABI, USA) on an ABI StepOnePlus qPCR instrument. The 20 μL reaction mixture was composed of 2 μL cDNA, 10 μL SybrGreen qPCR Master Mix, 0.4 μL upstream primer (10 μM), 0.4 μL downstream primer (10 μM) and 7.2 μL nuclease-free water. The qPCR conditions were as follows: staying at 95 °C for 3 min, then followed by 40 cycles of 95 °C for 7 s, 57 °C for 10 s and 72 °C for 15 s. Relative level of miR-21 was quantified via normalization to an endogenous control of U6 RNA. The primers used in this experiment were: miR-21 forward, 5'-ACA CTC CAG CTG GGT AGC TTA TCA GAC TG-3'; miR-21 reverse, 5'-CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG TCA ACA TCA-3'; U6 forward, 5'-CTC GCT TCG GCA GCA CA-3'; U6 reverse, 5'-AAC GCT TCA CGA ATT TGC GT-3'. We evaluated all the data with

respect to the miRNA expression by normalizing to the expression of U6 RNA and using the $2^{-\Delta\Delta Ct}$ method[S2].

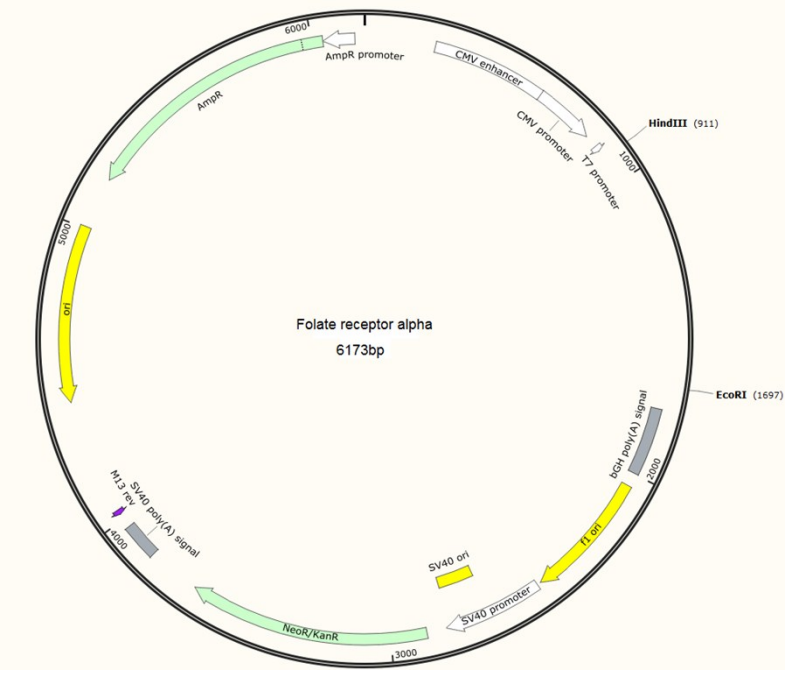
In vivo fluorescence imaging. Male athymic BALB/c (Balb/C-nu) mice were obtained from the Wuhan Servicebio Technology Co. Ltd. (Wuhan, China). The mice were 4-6 weeks old at the start of each experiment and weighed 20-25 g.

Four-week-old male BALB/c nude mice received a subcutaneous injection of 5×10^6 HeLa/MCF-7 cells into the underarm. Tumors were allowed to grow for 3-4 weeks to 1-2 cm in diameter. Before imaging, BALB/c nude mice, with or without tumors, were anesthetized with the combined use of tranquilizer and anesthetic. In detail, a 2 mg/kg dose of chlorpromazine hydrochloride was firstly injected intramuscularly, and a few minutes later, an intradermal injection was performed with an 80 mg/kg dose of pentobarbital sodium solution. Once the mice were anesthetized to be motionless, 1 mg/mL (200 μL) DNA probes were injected via the tail vein or 1 mg/mL (50 μL) DNA probes were in-situ injected into tumor-bearing mice. At specified times, fluorescence images of live mice were taken by IVIS Lumina III in vivo fluorescence imaging system (Caliper Life Sciences Inc.). A 640 nm (±25 nm) bandpass filter and a 680 nm long-pass filter were selected to be used as the excitation filter and the emission filter, respectively.

Table S1. Sequence of synthesized oligonucleotides.[a]

Name	Sequence (5'-3')
Ya	GAA TGC TGC GTG TAA TCC GTC TGT CCA CTG GCT ACT GTC
Yb	GAG TAC TAG AGG AAC ACG CAG CAT TCA CCT GTC TGT CGT
Yc	GGA CAG ACG GAT TTC CTC TAG TAC TCC TGT GTG ACT CCA
Hairpin probe H1	TCA ACA TCA GTC T**(Cy5)**G ATA AGC TAA CGT ACT AGC TTA T**(BHQ-2)**CA GAC TGA TTT TTT TTT TGA CAG TAG CCA GT
Hairpin probe H2	TAG CTT ATC AGA CTG ATG TTG ATC AGT CTG ATA AGC TAG TAC GTT TTT TTT TTT ACG ACA GAC AGG T
Hairpin probe H3	GAC CAC CGC ATC TCT GTG TGT AGC TCA GAT GCG GTG GTC CTT GAG AAT TTT TTT TTT GAC AGT AGC CAG T
Hairpin probe H4	TAG CTT ATC AGA CTG ATG TTG ATC AGT CTG ATA AGC TAG TAC GTT TTT TTT TTT ACG ACA GAC AGG T**(TAMRA)**
Linker L	**Alkynyl**-TTT TTT TTT TTG GAG TCA CAC AG
Synthetic miR-21	UAG CUU AUC AGA CUG AUG UUG A
Control RNA	GAG CUU AUC AGA AUC UCC AGG GG

[a] Ya, Yb and Yc form the Y-shaped scaffold with three sticky ends. Hairpin probes H1, H2 and targeting linker L conjugated with multivalent folate modifier peptide are assembled on the Y-shaped scaffold to form the tripartite DNA probe. Synthetic miR-21 is used as the initiator for HCR between probes H1 and H2 in in vitro experiments. Control RNA, the most homologous RNA to miR-21 in mammalian cells, is synthesized as the control to test the specificity of the designed HCR circuit.



Scheme S1. Constructed plasmid for genetic expression and the full protein sequence of the folate receptor alpha.

MAQRMTTQLLLLLVWVAVVGEAQTRIAWARTELLNVCMNAKHHKEKPGPEDKLHEQCRP
WRKNACCSTNTSQEAHKDVSYLYRFNWNHCGEMAPACKRHFIQDTCLYECSPNLGPWIQQ
VDQSWRKERVLNVPLCKEDCEQWWEDCRTSYTCKSNWHKGWNWTSGFNKCAVGAACQ
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GPWAAWPFLLSLALMLLWLLS

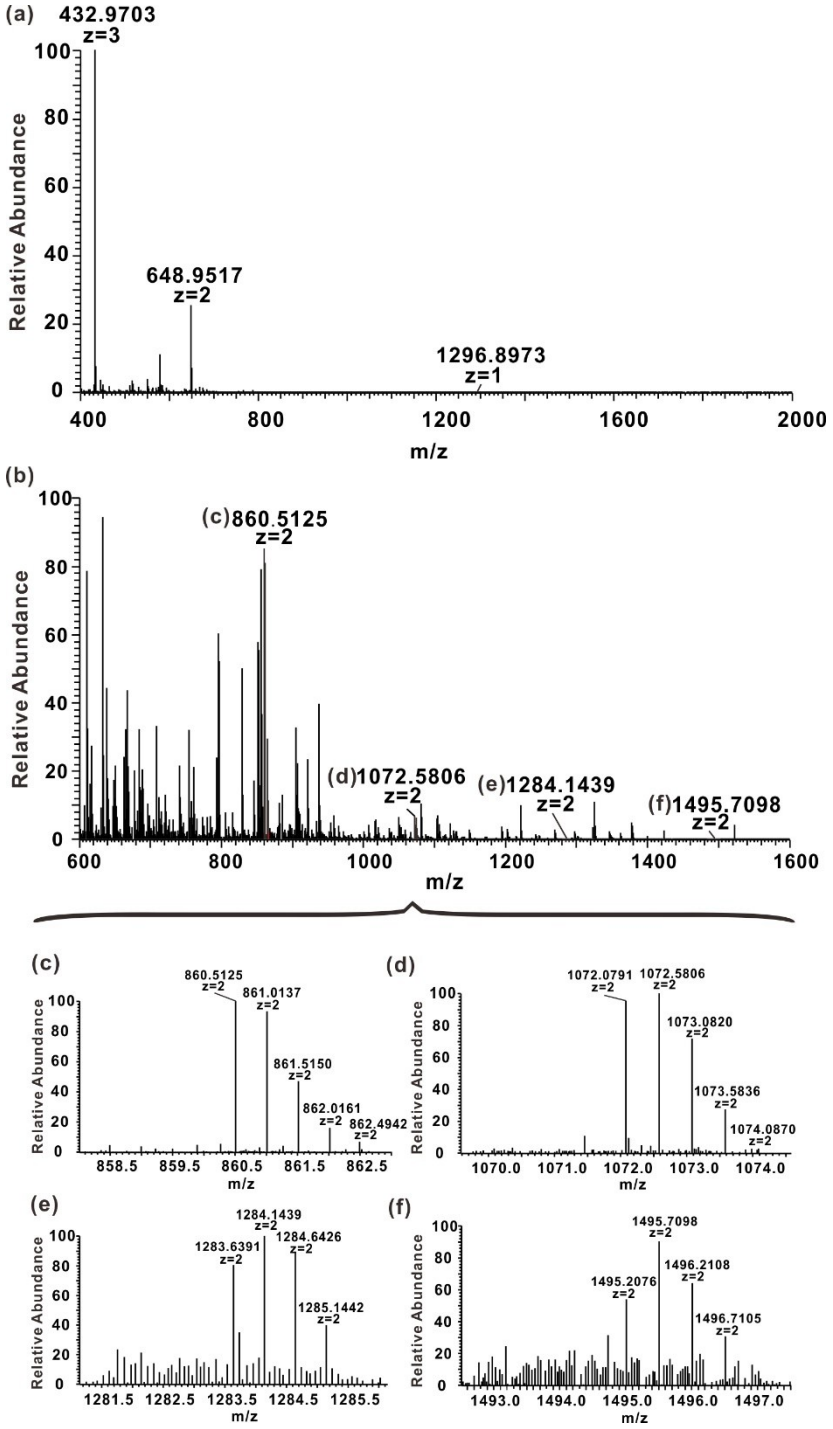

Figure S1. ESI MS spectrum of synthesized folate-conjugated peptide collected at positive ion mode. (a) The peak of peptide (m/z = 648.9517) corresponding to calculated value $[M+2H]^{2+}$ 648.9512. (b) The full spectrogram of peptide conjugated with one folate (m/z = 860.5125), two folates (m/z = 1072.0791), three folates (m/z = 1283.6391) and four folates (m/z = 1495.2076) corresponding to calculated value $[M+2H]^{2+}$ 860.5157, 1072.0803, 1283.6449, and 1495.2094, respectively. (c)-(f) Selective ion monitoring spectrogram for (b).

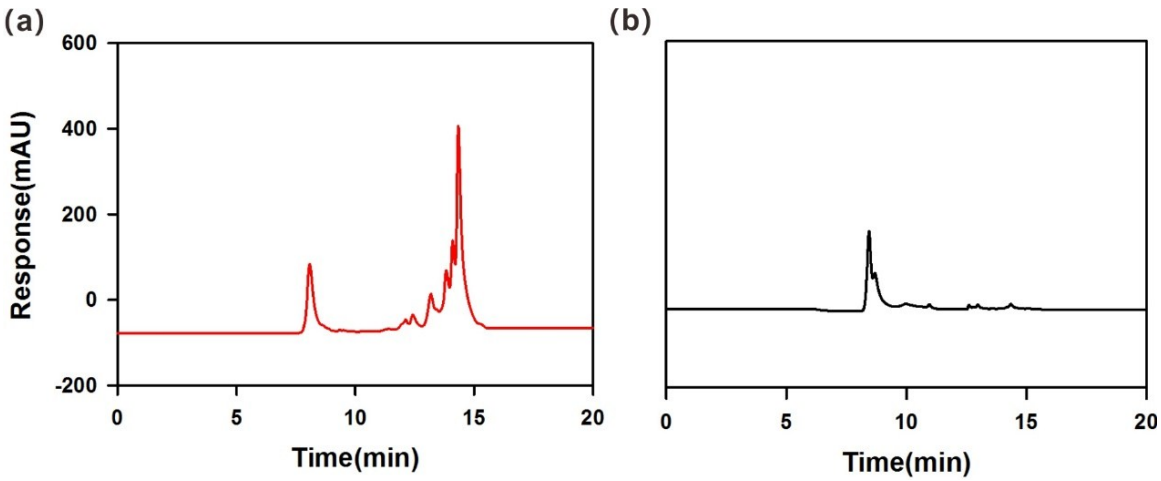

Figure S2. HPLC analysis of DNA Linker L modified with a folate-conjugated peptide (a) and the non-modified DNA Linker L (b). Ultraviolet absorption of DNA was collected at 260 nm on the detector of HPLC. The peak at 15 min in curve (a) represents the product of DNA Linker L with modification of folate-conjugated peptide, and the peak at about 8 min in curve (a) and (b) is the unreacted DNA Linker L.

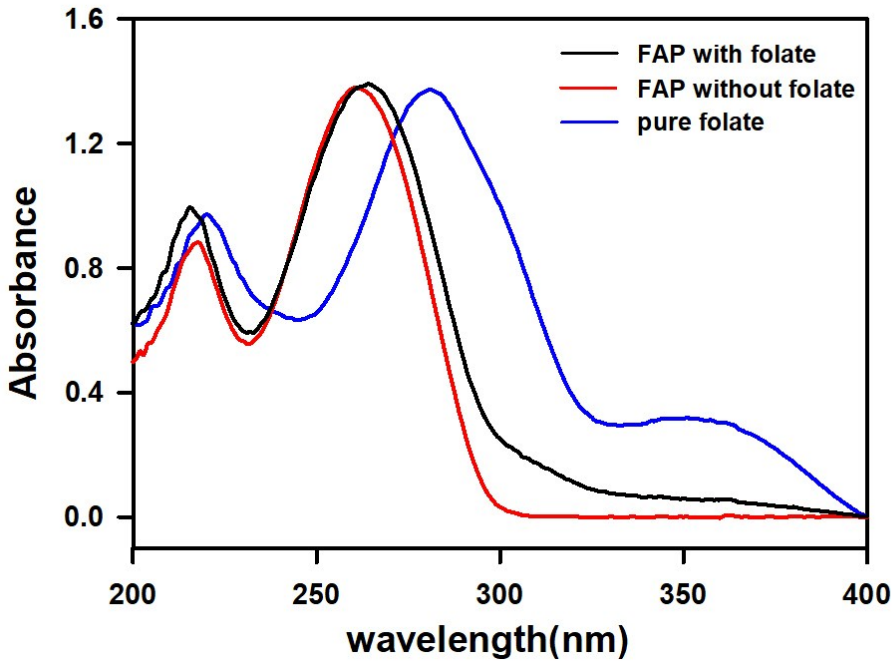

Figure S3. Normalized UV-vis spectra of DNA-peptide conjugate FAP probe with folate modification, without folate modification and pure folate solution. The UV absorption peaks of folate are obtained at 280 nm and 350 nm. The spectrum of FAP probe with folate modification shows increased absorption at 280 nm and 350 nm, which are indicators for the modification of folate.

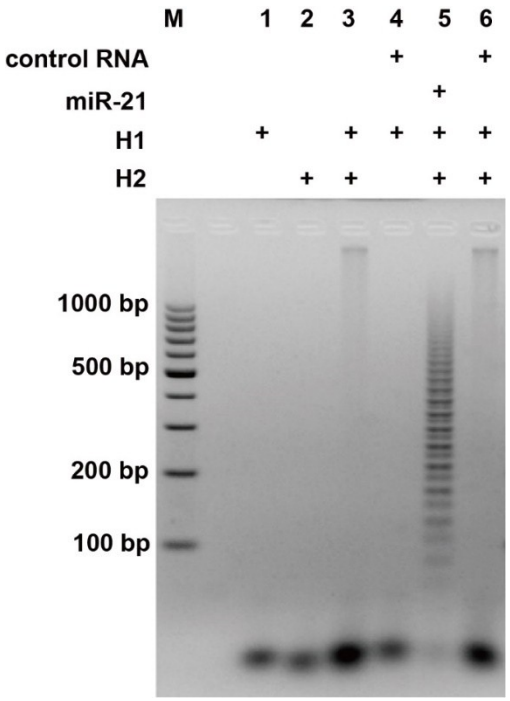

Figure S4. Agarose gel (4%) electrophoresis image for HCR design. The concentrations of hairpin probes H1 and H2 were 1 μM. The concentrations of control RNA and target miR-21 were 10 nM. M denotes molecular weight marker. The reaction time was three hours.

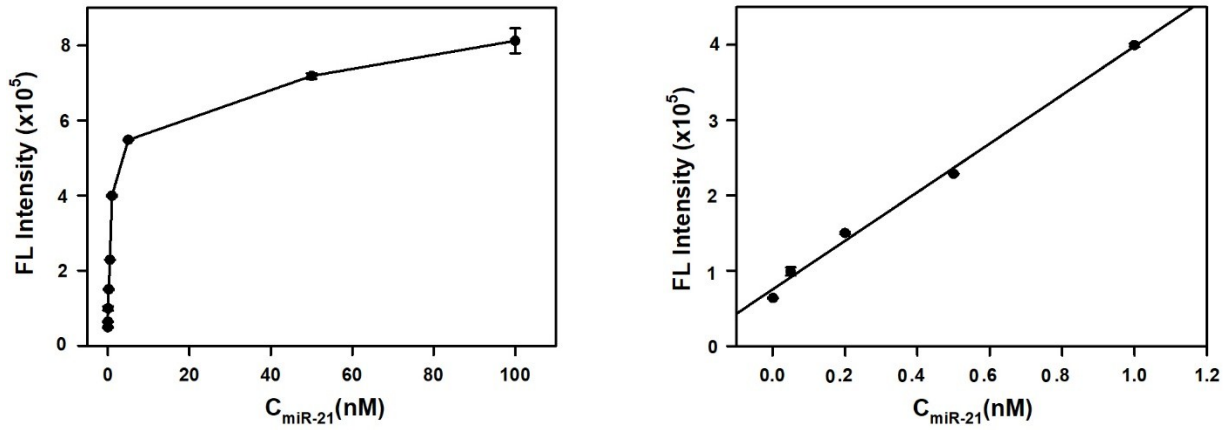

Figure S5. Plot of fluorescence peak intensities at 664 nm versus varying concentrations of miR-21. The excitation wavelength used in the assay was 635 nm. Error bars indicated SDs across three repetitive assays. The fluorescence emission intensity was found to be linear correlation to the concentration of miR-21 in the range of 0.001-1 nM and the limit of detection (LOD) was calculated to be 0.8 pM. The reaction time was three hours. The concentration of DNA probe was 100 nM.

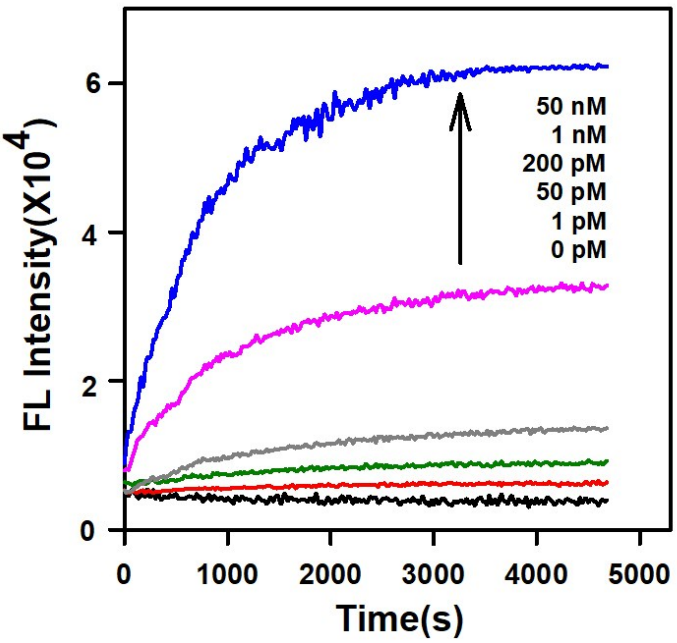

Figure S6. Real-time fluorescence signals for 100 nM Y-H1-H2-FAP probe to miR-21 of varying concentrations.

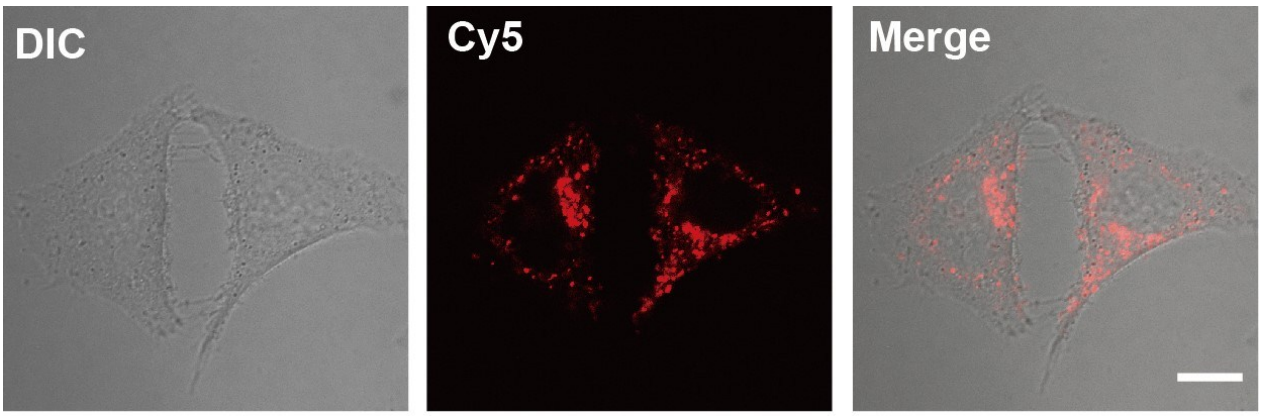

Figure S7. Fluorescence images at high magnification of HeLa cells incubated with 100 nM Y-H1-H2-FAP probe for three hours. Scale bar = 10 μm.

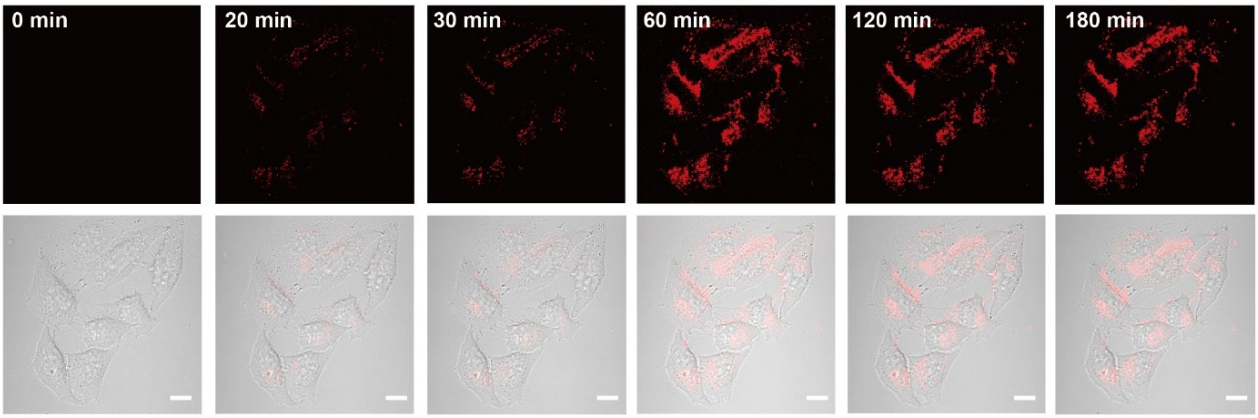

Figure S8. Real-time confocal fluorescence imaging of HeLa cells incubated with 100 nM Y-H1-H2-FAP probe. Scale bar = 10 μm.

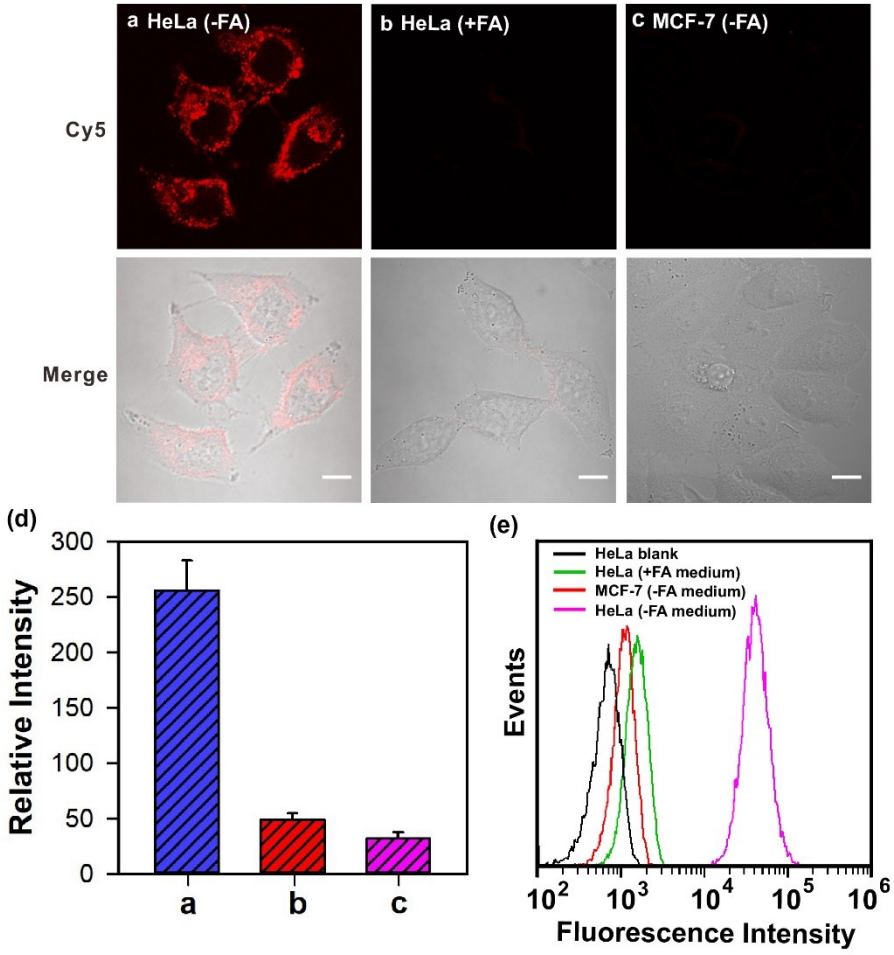

Figure S9. Fluorescence images of HeLa cells incubated with 100 nM Y-H1-H2-FAP probe in a culture medium without folate (a) or with folate (b), MCF-7 cells incubated with 100 nM Y-H1-H2-FAP probe in a culture medium without folate (c). (d) The mean fluorescence intensities of cells in panels a-c. (e) Flow cytometry analysis of cells in panels a-c. Scale bar = 10 μm.

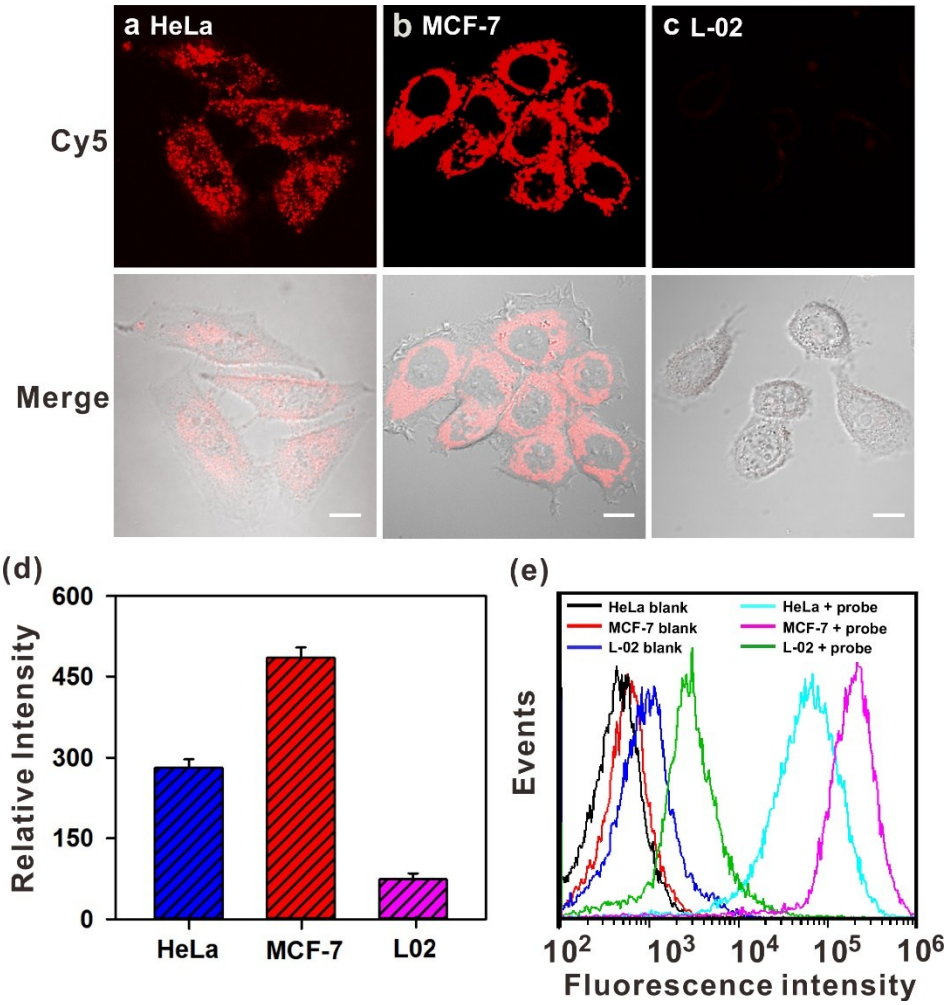

Figure S10. Fluorescence images of cells incubated with 100 nM Y-H1-H2-FAP probe. (a) HeLa cells, (b) FA receptor expressing plasmid transfected MCF-7 cells, (c) FA receptor expressing plasmid transfected L-02 cells. (d) Mean fluorescence intensities of cells in panels a-c. (e) Flow cytometry analysis of cells in panels a-c. Scale bar = 10 μm.

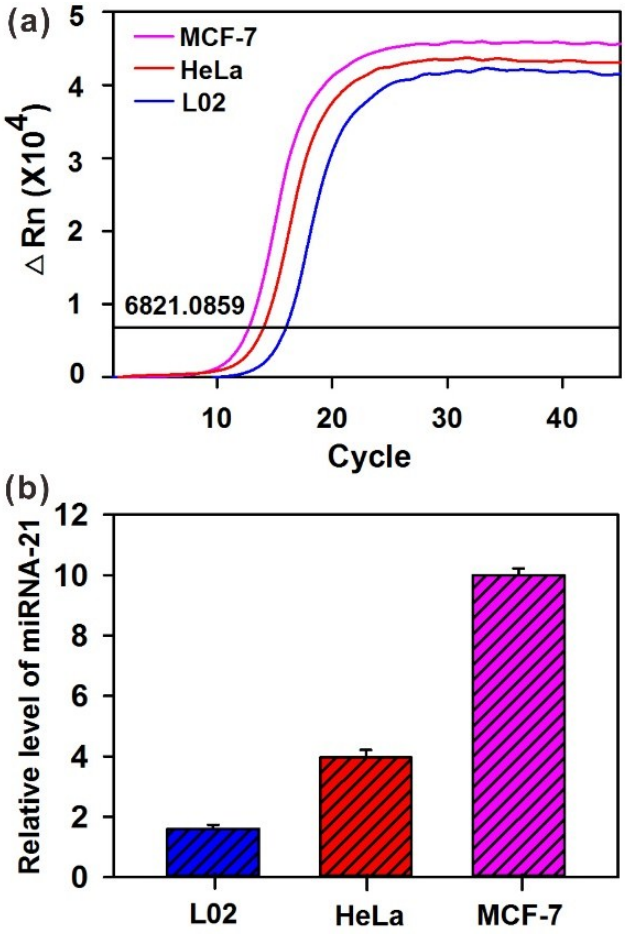

Figure S11. Expression analysis of miR-21 in MCF-7 (pink), HeLa (red) and L-02 (blue) cells. (a) Real-time fluorescence curves in qRT-PCR analysis. (b) Relative expression levels for miRNA-21. Error bars indicated SDs across three repetitive assays.

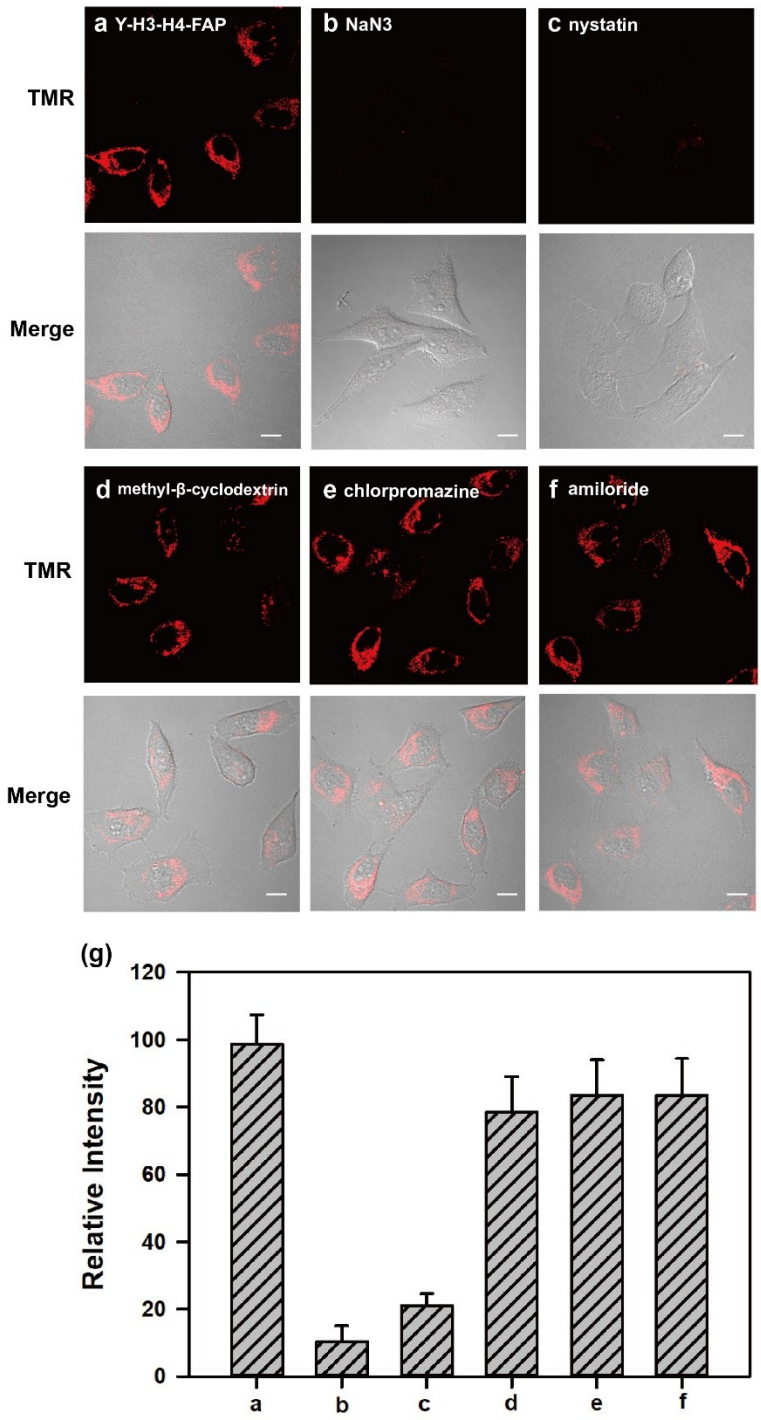

Figure S12. Fluorescence images of HeLa cells incubated with 100 nM Y-H3-H4-FAP probe at 37 ℃ (a), HeLa cells were pretreated using 1% NaN_3 (b), 50 μM nystatin (c), 10 mM methyl-β-cyclodextrin (d), 50 μM chlorpromazine (e) and 50 μM amiloride (f) followed by incubated with 100 nM Y-H3-H4-FAP probe at 37 ℃. (g) The mean fluorescence intensities of panels a-f. Scale bar = 10 μm.

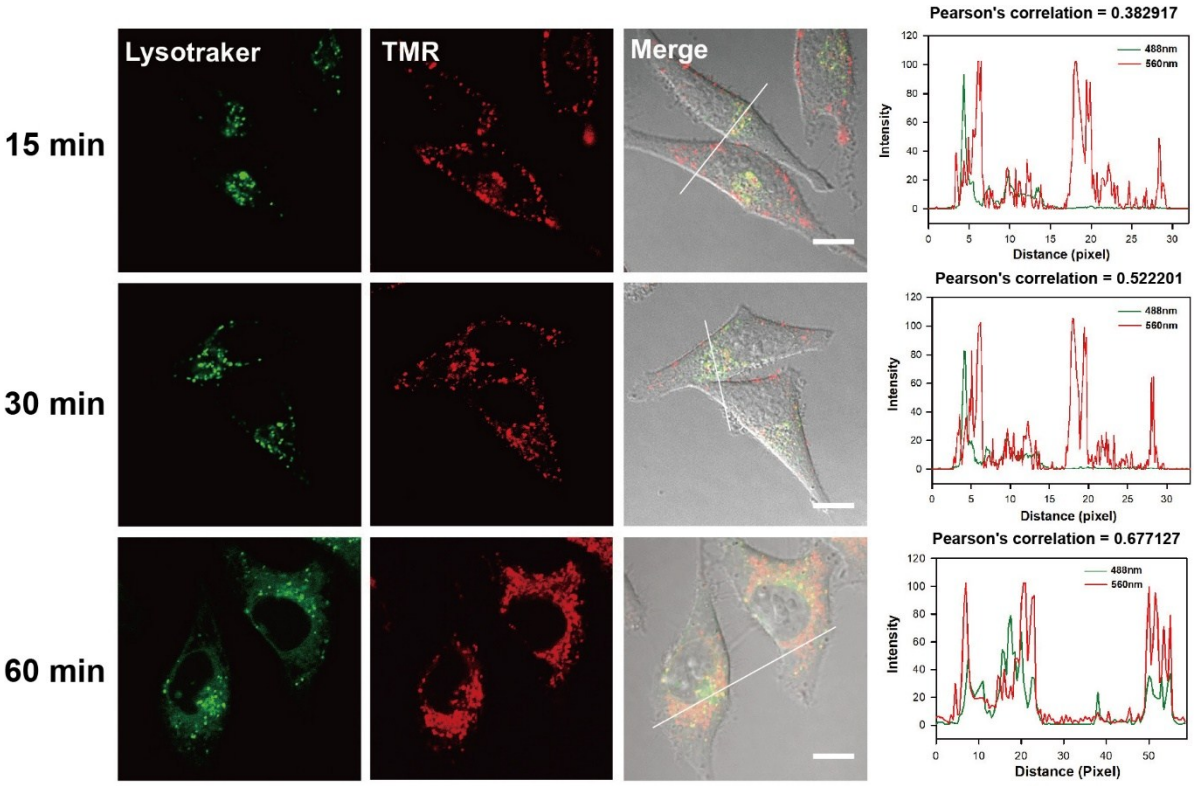

Figure S13. Real-time confocal fluorescence images of HeLa cells incubated with 100 nM Y-H3-H4-FAP probe. HeLa cells were stained with a culture medium containing 100 nM LysoTracker Green DND-26 for 20 min before imaging. The pictures in right side were channel intensities along the white line in the corresponding images. The red line represents 560 nm excitation channel and the green line represents 488 nm excitation channel. Scale bar = 10 μm.

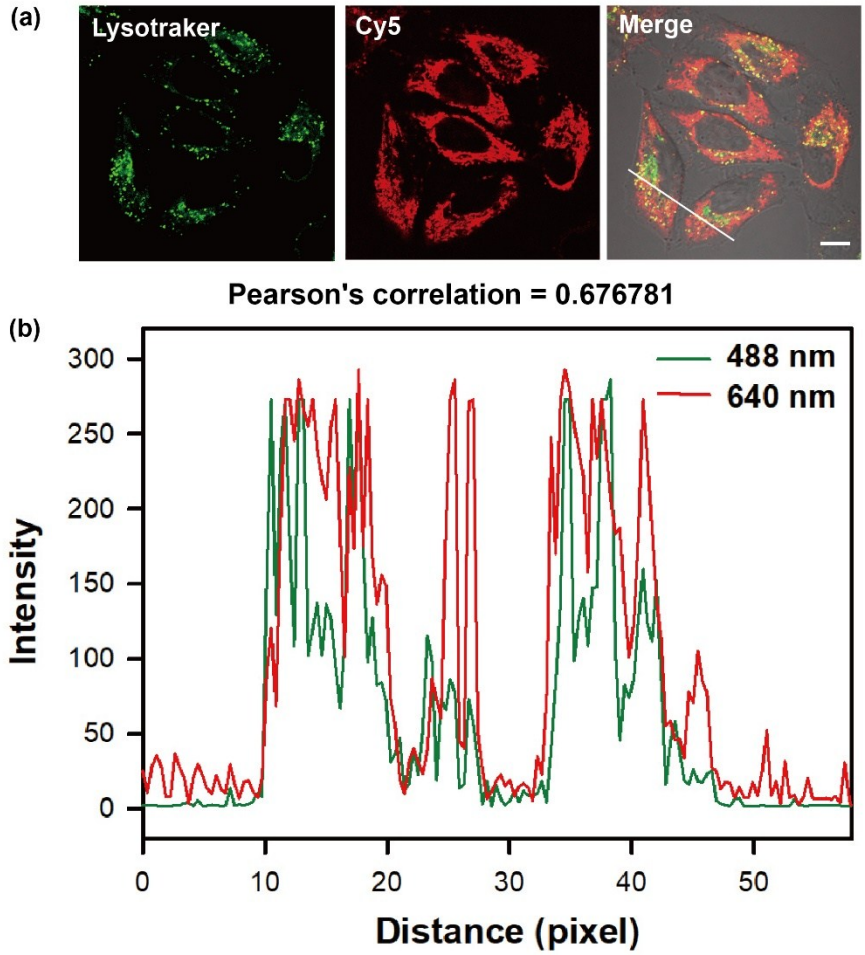

Figure S14. (a) Fluorescence images of HeLa cells after incubated for 3 h with 100 nM Y-H1-H2-FAP probe followed by staining with a culture medium containing 100 nM LysoTracker Green DND-26 for 20 min. (b) Channel intensities along the white line of (a). The red line represents 640 nm excitation channel and the green line represents 488 nm excitation channel. Scale bar = 10 μm.

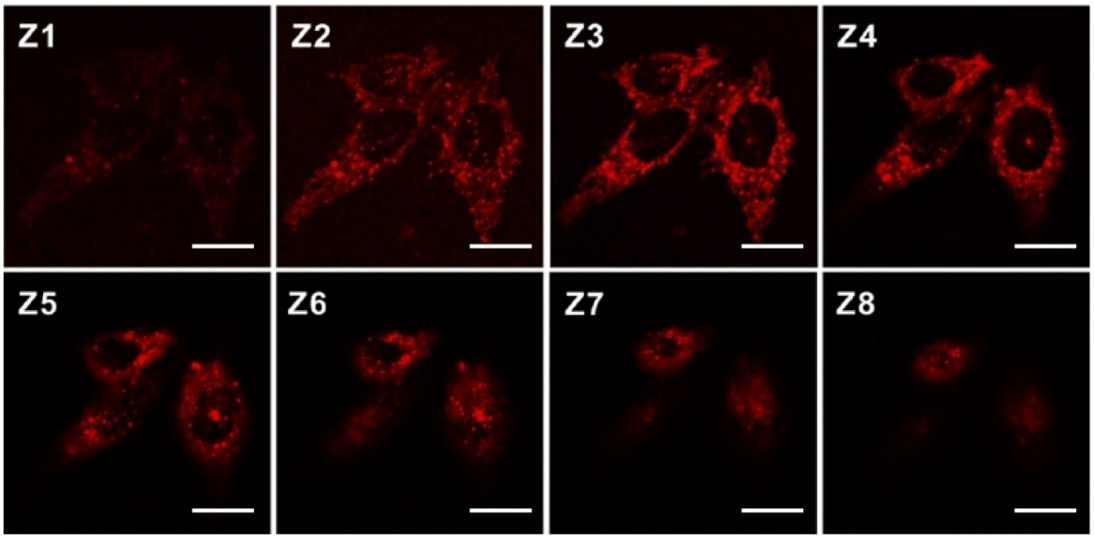

Figure S15. Z-axis images of the Cy5 fluorescence in the HeLa cells after incubated with 100 nM Y-H1-H2-FAP probe for 3 h. The images were taken at 1.38-μm z-axis intervals. Scale bar = 10 μm.

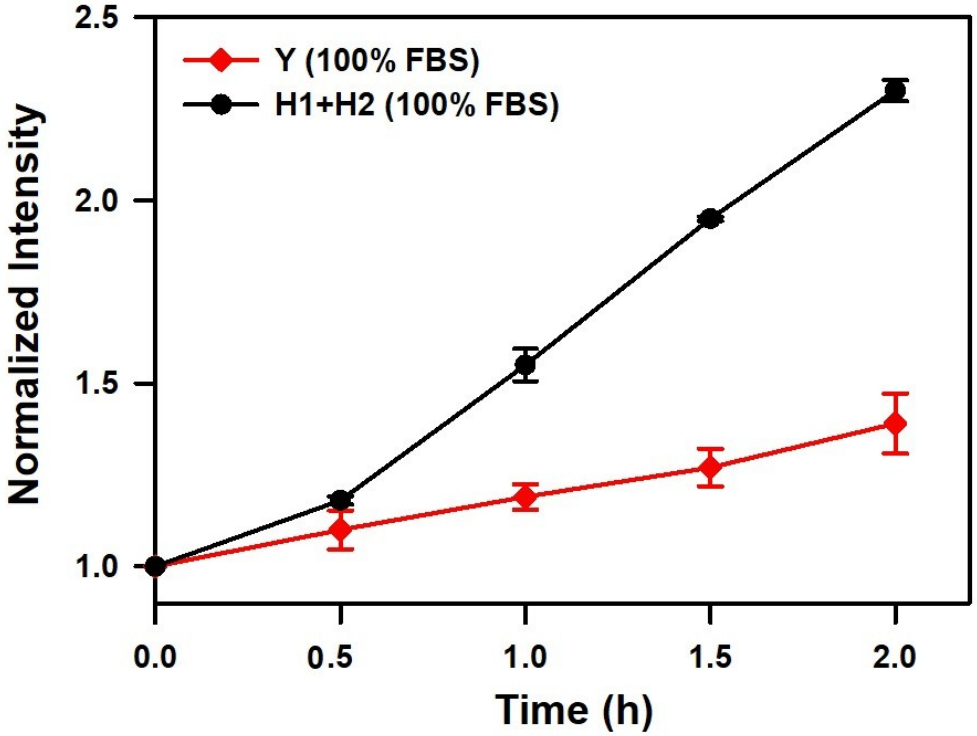

Figure S16. The stability analysis of 100 nM Y-H1-H2-FAP probe and 100 nM free hairpin H1 and H2 in 100% FBS. Error bars were obtained from three repetitive experiments.

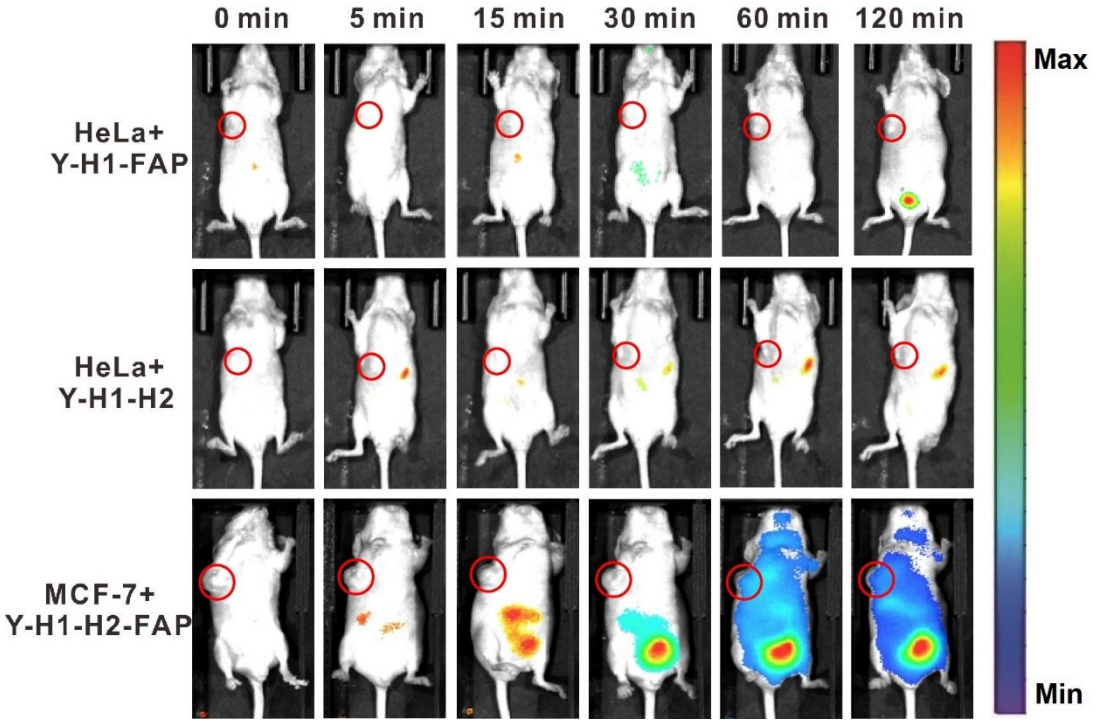

Figure S17. Time-dependent in vivo fluorescence imaging of the HeLa tumor-bearing mice injected via the tail vein with Y-H1-FAP probe and Y-H1-H2 probe, MCF-7 tumor-bearing mice injected with Y-H1-H2-FAP probe. Red circles represent tumor areas of mice and the fluorescence intensity of each point is taken from there.

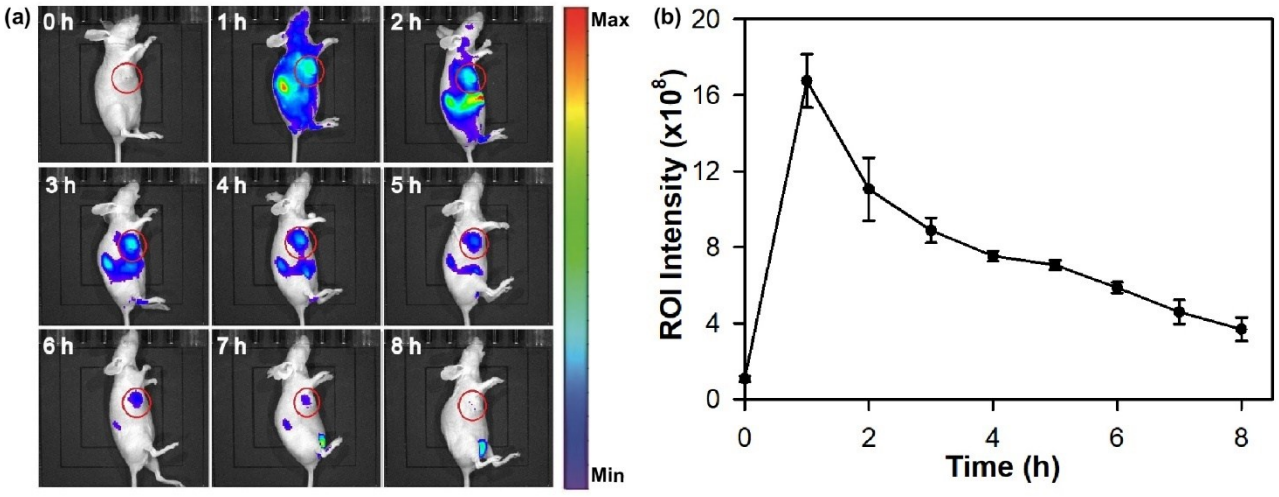

Figure S18. In vivo time-dependent fluorescence imaging (a) and normalized intensity analysis (b) of the HeLa tumor-bearing mice injected with Y-H1-H2-FAP probe for 8 h. Red circles represent tumor areas of mice and the fluorescence intensity of each point is taken from there. Error bars were obtained from three repetitive experiments.

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